

OLIGONUCLEOTIDE AND METHOD FOR DETECTING VEROTOXIN

## Field of the Invention

5 The present invention relates to oligonucleotides for use in detecting Verotoxin (hereafter, abbreviated as "VT") in clinical examinations, public health examinations, food evaluations and food poisoning examinations, as well as a detection method using said  
10 oligonucleotides. The oligonucleotide provided by the present invention can be used as a gene diagnosing reagent for cleaving, amplifying and detecting RNA or DNA, and is, for example, useful as a reagent for quantifying or diagnosing VT.

## Prior Art

15 Verotoxin is a potent toxin produced by Verotoxin-producing *Escherichia coli* (hereafter, abbreviated as "VTEC"), typically pathogenic *E. coli* O157. Although the primary symptom caused by infection with VTEC can be food poisoning represented by hemorrhagic colitis, it is  
20 reported that, in some cases, the symptom will advance to a hemolytic uremic syndrome (HUS) and, at worst, it will cause death.

Although VTEC has many various serotypes, which may  
25 be 60 types or more, in view of their detection frequencies, it is deemed that the major serotype is O157:H7. Further, VT includes VT type 1 which has the same structure as Shiga toxin produced by *Shigella dysenteriae* as well as VT type 2 having different  
30 physicochemical and immunological properties.

In Japan, VTEC mass infection occurs frequently and, therefore, in order to accomplish early detection and exclusion of the infectious source, speedy detection is desired. Further, from a clinical standpoint, since it  
35 is demonstrated that dosing with antibacterial composition comprising antibiotics at an early condition stage, i.e. within a few days from the onset of the

disease is effective, speedy identification of the bacteria is becoming important.

Means which had been used for examining VT include detection of O157 antigen. However, it is known that some *Salmonella* and *Citrobacter* strains show cross-antigenicity with O157 antigen, and it is reported that this detection method sometimes provides false positive results. In addition, mass infections caused by serotypes other than O157 have been reported, and therefore it is required to carry out tests using antisera against various serotypes.

Recently, a method of selectively detecting Verotoxin-producing bacteria comprising construction of oligonucleotides that selectively hybridize against a VTEC gene, and use of these oligonucleotides in a gene-amplification process (PCR process) as primers, has been proposed. However, since identification of the amplified DNA fragment is carried out with agarose electrophoresis, there remains a problem in view of the lack of speeds.

Contrary to the other types of food poisoning, VT results in a great deal of harm with smaller amounts of contaminating bacteria and, therefore, the food examination field, and the like, desire a more rapid and highly sensitive detection method. However, previous methods carry problems regarding their speed and simplicity. In addition, in order to simplify the examination, an examining instrument which carries out the detection automatically is desired.

It is known that when the target nucleic acid is RNA, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) can be used. This method involves synthesizing a cDNA from the target RNA in a reverse transcription step, and then amplifying a specific sequence of said cDNA by repetition of a cycle comprising heat denaturation, primer annealing and extension reactions, in the presence of a pair of primers complementarily and homologous to both ends of said specific sequence (the

antisense primer may be the same as the one used in reverse transcription step) as well as a thermostable DNA polymerase. However, RT-PCR method requires a two-step operation (a reverse transcription step and a PCR step) as well as an operation involving repetition of rapidly increasing and decreasing the temperature, which prevent its automation.

As amplification methods in cases where the target nucleic acid is RNA, in addition to the above, NASBA and 3SR method are known, whereby the specific sequence is amplified by the concerted action of a reverse transcriptase and an RNA polymerase. In these methods, the following procedures are carried out: using the target RNA as a template, a double-stranded DNA including a promoter sequence is synthesized with a primer containing the promoter sequence, reverse transcriptase and Ribonuclease H; this double-stranded DNA is used as a template in synthesizing an RNA containing the specific sequence with an RNA polymerase and, subsequently, this RNA provides a template in a chain reaction for synthesizing a double-stranded DNA containing the promoter sequence.

NASBA, 3SR, and the like, allow amplification at a constant temperature and are considered suitable for automation.

Because amplification methods such as NASBA and 3SR methods involve relatively low temperature reactions (41°C, for example), however, the target RNA may form an intramolecular structure that inhibits binding of the primer, which may reduce the reaction efficiency. Therefore, they require subjecting the target RNA to heat denaturation prior to the amplification reaction so as to destroy the intramolecular structure thereof and thus to improve the primer binding efficiency. As a result, the simplicity and speed of the methods are impaired.

Thus, an object of the present invention is to provide an oligonucleotide capable of complementarily

binding to an intramolecular structure-free region of the target RNA, the binding of which against the target RNA would not be inhibited even when being manipulated at relatively low temperature (for example, between 35 and 50°C, preferably, about 41°C), whereby its reaction efficiency would not be impaired. In particular, an object of the present invention is to provide an oligonucleotide capable of binding to the intramolecular structure-free region of VT1 RNA or VT2 RNA at relatively low temperature, or to provide an oligonucleotide primer which can be used in a nucleic acid amplification method so as to detect VT1 RNA or VT2 RNA, and also to provide simple, speedy and highly sensitive detecting method using such an oligonucleotide.

The invention according to claim 1 and intended to accomplish the objects relates to an oligonucleotide for detection or amplification of VT1 RNA, which oligonucleotide is capable of specifically binding to VT1 RNA, and comprises at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 5.

Moreover, the invention according to claim 2 and intended to accomplish the objects relates to an oligonucleotide for detection or amplification of VT2 RNA, which oligonucleotide is capable of specifically binding to VT2 RNA, and comprises at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 6 to 14.

Furthermore, the invention according to claim 3 and intended to accomplish the objects relates to a process of detecting VT1 RNA, wherein a specific sequence of VT1 RNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the formed RNA/DNA hybrid is digested by ribonuclease H to produce a single-stranded DNA, the single-stranded DNA is then used as a template for production of a double-stranded DNA having a promoter sequence capable of transcribing RNA comprising the

specific sequence or the sequence complementary to the  
specific sequence employing a DNA-dependent DNA  
polymerase, the double-stranded DNA produces an RNA  
transcription product in the presence of an RNA  
5 polymerase, and the RNA transcription product is then  
used as a template for cDNA synthesis employing the RNA-  
dependent DNA polymerase, the amplification process being  
characterized by employing a first oligonucleotide  
capable of specifically binding to VT1 RNA and comprising  
10 at least 10 contiguous bases of any of the sequences  
listed as SEQ. ID. Nos. 1 to 5 and a second  
oligonucleotide comprising at least 10 contiguous bases  
of any of the sequences listed as SEQ. ID. Nos. 15 to 18,  
where either the first or second oligonucleotide includes  
15 the RNA polymerase promoter sequence at the 5' end.

Still furthermore, the invention according to  
claim 4 and intended to accomplish the objects relates to  
a process of detecting VT2 RNA, wherein a specific  
sequence of VT2 RNA present in a sample is used as a  
20 template for synthesis of a cDNA employing an RNA-  
dependent DNA polymerase, the RNA of the formed RNA/DNA  
hybrid is digested by ribonuclease H to produce a single-  
stranded DNA, the single-stranded DNA is then used as a  
template for production of a double-stranded DNA having a  
25 promoter sequence capable of transcribing RNA comprising  
the specific sequence or the sequence complementary to  
the specific sequence employing a DNA-dependent DNA  
polymerase, the double-stranded DNA produces an RNA  
transcription product in the presence of an RNA  
30 polymerase, and the RNA transcription product is then  
used as a template for cDNA synthesis employing the RNA-  
dependent DNA polymerase, the amplification process being  
characterized by employing a first oligonucleotide  
capable of specifically binding to VT2 RNA, and  
35 comprising at least 10 contiguous bases of any of the  
sequences listed as SEQ. ID. Nos. 6 to 14 and a second  
oligonucleotide comprising at least 10 contiguous bases

of any of the sequences listed as SEQ. ID. Nos. 19 to 23, where either the first or second oligonucleotide includes the RNA polymerase promoter sequence at the 5' end.

5 The invention according to claim 5 relates to the process according to claim 3 or 4, wherein said amplification is carried out in the presence of an oligonucleotide probe capable of specifically binding to the RNA transcription product resulting from the amplification and labeled with an intercalator  
10 fluorescent pigment, and measuring changes in the fluorescent properties of the reaction solution. The invention according to claim 6 relates to the process according to claim 5, characterized in that the oligonucleotide probe is designed so as to  
15 complementarily bind to at least a portion of the sequence of the RNA transcription product, and the fluorescent property changes relative to that of a situation where a complex formation is absent. The invention according to claim 7 relates to the process  
20 according to claim 5 for detecting VT1 RNA, characterized in that the oligonucleotide probe comprises at least 10 contiguous bases of SEQ. ID. No. 24 or its complementary sequence. The invention according to claim 8 relates to the process according to claim 5 for  
25 detecting VT2 RNA, characterized in that the oligonucleotide probe comprises at least 10 contiguous bases of SEQ. ID. No. 25 or its complementary sequence. The present invention will be explained below.

30 First, the present invention provides an oligonucleotide useful in detecting VT1 RNA, which oligonucleotide is capable of specifically binding to VT1 RNA, and comprises at least 10 contiguous bases of any of the sequence listed as SEQ. ID. Nos. 1 to 5. This oligonucleotide is capable of binding to VT1 RNA at  
35 relatively low and constant temperature (35 to 50°C, preferably, about 41°C).

The RNA detecting process involving the step of

amplifying VT1 RNA in a sample provided by the present invention includes PCR method, NASBA method, 3SR method, or the like. However, it is preferred that the nucleic acid amplification is a one which can be conducted under  
5 constant temperature, such as NASBA or 3SR method in which specific sequence within VT1 RNA is amplified with the concerted action of reverse transcriptase and RNA polymerase.

For example, in the NASBA method, a specific  
10 sequence of VT1 RNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the RNA/DNA hybrid is digested by ribonuclease H to produce a single-stranded DNA, the single-stranded DNA is then used as a  
15 template for production of a double-stranded DNA having a promoter sequence capable of transcribing RNA comprising the specific sequence or the sequence complementary to the specific sequence employing a DNA-dependent DNA polymerase, the double-stranded DNA produces an RNA  
20 transcription product in the presence of an RNA polymerase, and the RNA transcription product is then used as a template for cDNA synthesis employing the RNA-dependent DNA polymerase. The process of the present invention is characterized by employing a first  
25 oligonucleotide capable of specifically binding to VT1 RNA and comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 1 to 5 and a second oligonucleotide comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 15 to 18  
30 and having a sequence homologous to a portion of the VT1 RNA sequence to be amplified, where either the first or second oligonucleotide includes the RNA polymerase promoter sequence at the 5' end.

Although the RNA-dependent DNA polymerase, the DNA-  
35 dependent DNA polymerase and the ribonuclease H are not critical, AMV reverse transcriptase that has all of these types of activity is most preferably used. Moreover,

although the RNA polymerase is not critical, T7 phage RNA polymerase or SP6 phage RNA polymerase is preferably used.

In the above amplification process, an  
5 oligonucleotide that is complementary to the region  
adjacent to and overlapping with the 5' end region of the  
specific sequence (bases 1 to 10) of VT1 RNA sequence is  
added, and the VT1 RNA is cleaved (with ribonuclease H)  
at the 5' end region of the specific sequence to provide  
10 the initial template for nucleic acid amplification,  
thereby allowing amplification of VT1 RNA even when the  
specific sequence is not positioned at the 5' end. The  
oligonucleotide used for this cleaving may, for example,  
be any of those of SEQ. ID. Nos. 1 to 5, provided that it  
15 differs from the one used as the first oligonucleotide in  
the amplification process. In addition, the  
oligonucleotide for cleaving is preferably chemically  
modified (for example, aminated) at the 3' hydroxyl group  
in order to prevent an extension reaction from the  
20 3' end.

Although the RNA transcription product obtained by  
the above nucleic acid amplification can be detected by a  
known method, per se, preferably, it is detected by  
carrying out the above amplification process in the  
25 presence of an oligonucleotide probe labeled with an  
intercalator fluorescent pigment, and measuring changes  
in the fluorescent properties of the reaction solution.  
Examples of the oligonucleotide probe include one in  
which the intercalator fluorescent pigment is bonded to a  
30 phosphorus atom in the oligonucleotide through a linker.  
The probe is characterized in that when it forms a  
double-stranded chain with the target nucleic acid  
(complementary nucleic acid), separation analysis is not  
required because the intercalator portion intercalates  
35 into the double-stranded chain portion to vary the  
fluorescent characteristics (Ishiguro, T. et al. (1996),  
Nucleic Acids Res. 24 (24) 4992 - 4997).



The probe sequence is not critical so long as it has a sequence complementary to at least a portion of the RNA transcription product. However, the probe sequence is preferably one comprising at least 10 contiguous bases of the sequence listed as SEQ. ID. No. 24. Moreover, chemical modification (for example, glycolic acid addition) at the 3' end hydroxyl group of the probe is preferred in order to inhibit an extension reaction in which the probe acts as a primer.

It becomes possible to amplify and detect RNA comprising the same sequence as the specific sequence of VT1 RNA in a single tube at a constant temperature and in a single step by carrying out the amplification process in the presence of the probe, as explained above, and, thus, the amplification process is easily automated.

Next, the present invention provides an oligonucleotide useful in detecting VT2 RNA, which oligonucleotide is capable of specifically binding to VT2 RNA, and comprises at least 10 contiguous bases of any of the sequence listed as SEQ. ID. Nos. 6 to 14. This oligonucleotide is capable of binding to VT2 RNA at relatively low and constant temperature (35 to 50°C, preferably, about 41°C).

The RNA detecting process involving the step of amplifying VT2 RNA in a sample provided by the present invention includes PCR method, NASBA method, 3SR method, or the like. However, it is preferred that the nucleic acid amplification is a one which can be conducted under constant temperature, such as NASBA or 3SR method in which specific sequence within VT2 RNA is amplified with the concerted action of reverse transcriptase and RNA polymerase.

For example, in the NASBA method, a specific sequence of VT2 RNA present in a sample is used as a template for synthesis of a cDNA employing an RNA-dependent DNA polymerase, the RNA of the RNA/DNA hybrid is digested by ribonuclease H to produce a single-

stranded DNA, the single-stranded DNA is then used as a template for production of a double-stranded DNA having a promoter sequence capable of transcribing RNA comprising the specific sequence or the sequence complementary to the specific sequence employing a DNA-dependent DNA polymerase, the double-stranded DNA produces an RNA transcription product in the presence of an RNA polymerase, and the RNA transcription product is then used as a template for cDNA synthesis employing the RNA-dependent DNA polymerase. The process of the present invention is characterized by employing a first oligonucleotide capable of specifically binding to VT2 RNA and comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 6 to 14 and a second oligonucleotide comprising at least 10 contiguous bases of any of the sequences listed as SEQ. ID. Nos. 19 to 23 and having a sequence homologous to a portion of the VT2 RNA sequence to be amplified, where either the first or second oligonucleotide includes the RNA polymerase promoter sequence at the 5' end.

Although the RNA-dependent DNA polymerase, the DNA-dependent DNA polymerase and the ribonuclease H are not critical, AMV reverse transcriptase that has all of these types of activity is most preferably used. Moreover, although the RNA polymerase is not critical, T7 phage RNA polymerase or SP6 phage RNA polymerase is preferably used.

In the above amplification process, an oligonucleotide that is complementary to the region adjacent to and overlapping with the 5' end region of the specific sequence (bases 1 to 10) of VT2 RNA sequence is added, and the VT2 RNA is cleaved (with ribonuclease H) at the 5' end region of the specific sequence to give the initial template for nucleic acid amplification, thereby allowing amplification of VT2 RNA even when the specific sequence is not positioned at the 5' end. The oligonucleotide used for this cleaving may, for example,

be any of those of SEQ. ID. Nos. 6 to 14, provided that it differs from the one used as the first oligonucleotide in the amplification process. In addition, the oligonucleotide for cleaving is preferably chemically modified (for example, aminated) at the 3' hydroxyl group in order to prevent an extension reaction from the 3' end.

Although the RNA transcription product obtained by the above nucleic acid amplification can be detected by a method known per se, preferably, it is detected by carrying out the above amplification process in the presence of an oligonucleotide probe labeled with an intercalator fluorescent pigment, and measuring changes in the fluorescent properties of the reaction solution. Examples of the oligonucleotide probe include one in which the intercalator fluorescent pigment is bonded to a phosphorus atom in the oligonucleotide through a linker. The probe is characterized in that when it forms a double-stranded chain with the target nucleic acid (complementary nucleic acid), separation analysis is not required because the intercalator portion intercalates into the double-stranded chain portion to vary the fluorescent characteristics (Ishiguro, T. et al. (1996), Nucleic Acids Res. 24 (24) 4992 - 4997).

The probe sequence is not critical so long as it has a sequence complementary to at least a portion of the RNA transcription product. However, the probe sequence is preferably one comprising at least 10 contiguous bases of the sequence listed as SEQ. ID. No. 25. Moreover, chemical modification (for example, glycolic acid addition) at the 3' end hydroxyl group of the probe is preferred in order to inhibit an extension reaction based on the probe used as a primer.

It becomes possible to amplify and detect RNA comprising the same sequence as the specific sequence of VT2 RNA in a single tube at a constant temperature and in a single step by carrying out the amplification process

in the presence of the probe, as explained above, and, thus, the amplification process is easily automated.

#### Brief Description of the Drawings

Fig. 1 is a urea modified 6% polyacrylamide electrophoresis diagram for samples obtained by performing cleaving experiments on VT1 RNA standard at 41°C, using Oligos 1 to 6 and AMV-Reverse Transcriptase (black and white inverted). The lanes without any indications are unrelated to the present invention.

Fig. 2 is a urea modified 6% polyacrylamide electrophoresis diagram for samples obtained by performing cleaving experiments on VT2 RNA standard at 41°C, using Oligos 7 to 15 and AMV-Reverse Transcriptase (black and white inverted). The lanes without any indications are unrelated to the present invention.

Fig. 3 is a 4% agarose gel electrophoresis diagram for RNA amplification reactions of VT1 RNA standard performed as described in Example 3 using oligonucleotide probe combinations (a) to (c) shown in Table 3 (black and white inverted), with an initial RNA amount of  $10^4$  copies/30  $\mu$ l and  $10^3$  copies/30  $\mu$ l. Lane 1 is the result for combination (a) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 2 and 3 are for combination (a) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 4 is for combination (a) using only the diluent instead of RNA samples (control); lane 5 is the result for combination (b) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 6 and 7 are for combination (b) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 8 is for combination (b) using only the diluent instead of RNA samples (control); lane 9 is the result for combination (c) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 10 and 11 are for combination (c) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; and lane 12 is for combination (c) using only the diluent instead of RNA samples (control).

Specific bands were confirmed in every combination.

Fig. 4 is a 4% agarose gel electrophoresis diagram for RNA amplification reactions of VT1 RNA standard performed as described in Example 3 using oligonucleotide probe combinations (d) to (f) shown in Table 3 (black and white inverted), with an initial RNA amount of  $10^4$  copies/30  $\mu$ l and  $10^3$  copies/30  $\mu$ l. Lane 1 is the result for combination (d) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 2 and 3 are for combination (d) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 4 is for combination (d) using only the diluent instead of RNA samples (control); lane 5 is the result for combination (e) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 6 and 7 are for combination (e) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 8 is for combination (e) using only the diluent instead of RNA samples (control); lane 9 is the result for combination (f) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 10 and 11 are for combination (f) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; and lane 12 is for combination (f) using only the diluent instead of RNA samples (control). Specific bands were confirmed in every combination.

Fig. 5 is a 4% agarose gel electrophoresis diagram for RNA amplification reactions of VT2 RNA standard performed as described in Example 4 using oligonucleotide probe combinations (g) to (i) shown in Table 4 (black and white inverted), with an initial RNA amount of  $10^4$  copies/30  $\mu$ l and  $10^3$  copies/30  $\mu$ l. Lane 1 is the result for combination (g) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 2 and 3 are for combination (g) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 4 is for combination (g) using only the diluent instead of RNA samples (control); lane 5 is the result for combination (h) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l;

lanes 6 and 7 are for combination (h) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 8 is for combination (h) using only the diluent instead of RNA samples (control); lane 9 is the result for combination (i) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 10 and 11 are for combination (i) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; and lane 12 is for combination (i) using only the diluent instead of RNA samples (control). Specific bands were confirmed in every combination.

Fig. 6 is a 4% agarose gel electrophoresis diagram for RNA amplification reactions of VT2 RNA standard performed as described in Example 4 using oligonucleotide probe combinations (j) to (l) shown in Table 4 (black and white inverted), with an initial RNA amount of  $10^4$  copies/30  $\mu$ l and  $10^3$  copies/30  $\mu$ l. Lane 1 is the result for combination (j) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 2 and 3 are for combination (j) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 4 is for combination (j) using only the diluent instead of RNA samples (control); lane 5 is the result for combination (k) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 6 and 7 are for combination (k) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; lane 8 is for combination (k) using only the diluent instead of RNA samples (control); lane 9 is the result for combination (l) with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 10 and 11 are for combination (l) with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; and lane 12 is for combination (l) using only the diluent instead of RNA samples (control). Specific bands were confirmed in every combination.

Fig. 7 is a 4% agarose gel electrophoresis diagram for RNA amplification reactions of VT2 RNA standard performed as described in Example 4 using oligonucleotide probe combination (m) shown in Table 4 (black and white

inverted), with an initial RNA amount of  $10^4$  copies/30  $\mu$ l and  $10^3$  copies/30  $\mu$ l. Lane 1 is the result with an initial RNA amount of  $10^4$  copies/30  $\mu$ l; lanes 2 and 3 are the results with an initial RNA amount of  $10^3$  copies/30  $\mu$ l; and lane 4 is the result obtained by using only the diluent instead of RNA samples (control). Specific bands were confirmed in every combination.

Fig. 8 shows the results obtained in Example 5 for samples prepared from the VT2 RNA standard with an initial RNA amount of from  $10^1$  copies/30  $\mu$ l to  $10^5$  copies/30  $\mu$ l. Panel (a) is a fluorescence profile exhibiting the fluorescence increase ratio that increases with the reaction time-course formation of RNA. Panel (b) is a calibration curve exhibiting the relationship between the logarithm of the initial RNA amount and the detection time (time at which the relative fluorescence reaches 1.2).  $\square$  shows the result for  $10^5$  copies,  $\circ$  is for  $10^4$  copies,  $\triangle$  is for  $10^3$  copies,  $\diamond$  is for  $10^2$  copies, + is for 10 copies and  $\times$  is for control. It was demonstrated that RNA with initial copies of  $10^1$  copies/30  $\mu$ l can be detected by a reaction for about 20 minutes, and that there is a correlation between the initial RNA amount and the detection time.

#### Examples

The present invention will now be explained in greater detail by way of examples, with the understanding that the invention is not limited by these examples.

#### Example 1

(1) An oligonucleotide which specifically binds to VT1 RNA at  $41^\circ\text{C}$  was selected. A standard RNA comprising a region of base Nos. 228 to 1558 of the VT1 RNA base sequence (Calderwood, S. B. et al., Proc. Natl. Acad. Sci. U.S.A., 84, 4364 - 4368 (1987), US GenBank Registered No. M16625) was quantified by ultraviolet absorption at 260 nm, and then diluted to a concentration

of 1.33 pmol/ $\mu$ l with an RNA diluent (10 mM Tris-HCl (pH 8.0)), 0.1 mM EDTA, 1 mM DTT, 0.5 U/ $\mu$ l RNase Inhibitor).

- 5 (2) 14.0  $\mu$ l of a reaction solution having the following composition was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tube™, Perkin-Elmer Co. Ltd.)

Reaction Solution Composition

- 10 60.0 mM Tris-HCl buffer (pH 8.6)  
90.0 mM potassium chloride  
13.0 mM magnesium chloride  
1.0 mM DTT  
80.0 nM standard RNA  
0.8  $\mu$ M oligonucleotide (one of the oligonucleotides  
15 shown below).  
Oligo-1: SEQ. ID. No. 1;  
Oligo-2: SEQ. ID. No. 2;  
Oligo-3: SEQ. ID. No. 26;  
Oligo-4: SEQ. ID. No. 3;  
20 Oligo-5: SEQ. ID. No. 4;  
Oligo-6: SEQ. ID. No. 5

Distilled water for adjusting volume

- (3) The reaction solutions were then incubated at 41°C for 5 minutes, and then 1  $\mu$ l of 8.0 U/ $\mu$ l AMV-Reverse  
25 Transcriptase (Takara Shuzo Co. Ltd.; an enzyme which cleaves RNA of a double stranded-DNA/RNA) was added thereto.

- (4) Subsequently, the PCR tubes were incubated at 41°C for 10 minutes. Modified-urea polyacrylamide gel  
30 (acrylamide concentration: 6%; urea: 7M) electrophoresis was conducted to confirm the cleaved fragments after the reaction. Dyeing following the electrophoresis was carried out with SYBR Green II™ (Takara Shuzo Co. Ltd.). Upon binding of the  
35 oligonucleotide to the specific site of the target RNA,



RNA of the double stranded DNA/RNA is cleaved by the ribonuclease H activity of AMV-Reverse Transcriptase and, thereby, a characteristic band can be observed.

(5) The results of the electrophoresis are shown in Fig. 1 (black and white inverted). If the oligonucleotide binds specifically to the standard RNA, the standard RNA will be cleaved at this region, yielding a decomposition product having a characteristic chain length. Table 1 shows the positions of the standard RNA where each oligonucleotide had specifically bind and the expected band lengths of the fragments. Cleavages at the expected positions were confirmed with Oligos 1 to 6. These indicated that these oligonucleotides bind strongly to the VT1 RNA under a constant temperature of 41°C.

Table 1

Oligo	Position <sup>1)</sup>	Expected band Length (base)
Oligo -1	425	425, 912
Oligo -2	555	555, 782
Oligo -3	710	710, 627
Oligo -4	890	890, 447
Oligo -5	980	980, 357
Oligo -6	1031	1031, 306

1) The position designates the 5' end number of the oligonucleotide which binds to the VT1 RNA standard (1337 base).

Example 2

(1) An oligonucleotide which specifically binds to VT2 RNA at 41°C was selected. A standard RNA comprising a region of base Nos. 81 to 1437 of the VT2 RNA base sequence (Schmitt, C. K. et al., Infect. Immun, 59, 1065 - 1073 (1991), US GenBank Registered No. X07865) was quantified by ultraviolet absorption at 260 nm, and then diluted to a concentration of 1.75 pmol/μl with an RNA diluent (10 mM Tris-HCl (pH 8.0)), 0.1 mM EDTA, 1 mM DTT, 0.5 U/μl RNase Inhibitor).

(2) 14.0 μl of a reaction solution having the following composition was dispensed into 0.5 ml volume

PCR tubes (Gene Amp Thin-Walled Reaction Tube™, Perkin-Elmer Co. Ltd.)

Reaction Solution Composition

60.0 mM Tris-HCl buffer (pH 8.6)

5 90.0 mM potassium chloride

13.0 mM magnesium chloride

1.0 mM DTT

80.0 nM standard RNA

10 0.8 μM oligonucleotide (one of the oligonucleotides shown below).

Oligo-7: SEQ. ID. No. 6;

Oligo-8: SEQ. ID. No. 7;

Oligo-9: SEQ. ID. No. 8;

15 Oligo-10: SEQ. ID. No. 9;

Oligo-11: SEQ. ID. No. 10;

Oligo-12: SEQ. ID. No. 11;

Oligo-13: SEQ. ID. No. 12;

Oligo-14: SEQ. ID. No. 13;

Oligo-15: SEQ. ID. No. 14;

20 Distilled water for adjusting volume

(3) The reaction solutions were then incubated at 41°C for 5 minutes, and then 1 μl of 8.0 U/μl AMV-Reverse Transcriptase (Takara Shuzo Co. Ltd.; an enzyme which cleaves RNA of a double stranded-DNA/RNA) was added thereto.

(4) Subsequently, the PCR tubes were incubated at 41°C for 10 minutes. Modified-urea polyacrylamide gel (acrylamide concentration: 6%; urea: 7M) electrophoresis was conducted to confirm the cleaved fragments after the reaction. Dyeing following the electrophoresis was carried out with SYBR Green II™ (Takara Shuzo Co. Ltd.). Upon binding of the oligonucleotide to the specific site of the target RNA, RNA of the double stranded DNA/RNA is cleaved by the ribonuclease H activity of AMV-Reverse Transcriptase and, thereby, a characteristic band can be observed.

(5) The results of the electrophoresis are shown in Fig. 2 (black and white inverted). If the oligonucleotide binds specifically to the standard RNA, the standard RNA will be cleaved at this region, yielding a decomposition product having a characteristic chain length. Table 2 shows the positions of the standard RNA where each oligonucleotide had specifically bound and the expected band lengths of the fragments. Cleavages at the expected positions were confirmed with Oligos 7 to 15. These indicated that these oligonucleotides bind strongly to the VT2 RNA under a constant temperature of 41°C.

Table 2

Oligo	Position <sup>1)</sup>	Expected band Length (base)
Oligo -7	102	102, 1259
Oligo -8	260	260, 1101
Oligo -9	365	365, 996
Oligo -10	436	436, 925
Oligo -11	675	675, 686
Oligo -12	723	723, 638
Oligo -13	787	787, 574
Oligo -14	848	848, 513
Oligo -15	986	986, 375

1) The position designates the 5' end number of the oligonucleotide which binds to the VT2 RNA standard (1361 base).

### Example 3

RNA amplification reactions were carried out using the oligonucleotides which specifically bind to VT1 RNA.

(1) As described in example 1, VT1 standard RNA was diluted to  $10^4$  copies/2.5  $\mu$ l and  $10^3$  copies/2.5  $\mu$ l with an RNA diluent (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 U/ $\mu$ l RNase Inhibitor (Takara Shuzo Co. Ltd.), 5 mM DTT). In the control test sections (negative), only the diluent was used.

(2) 23.3  $\mu$ l of a solution having the following composition was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tube™, Perkin-Elmer Co. Ltd.), followed by addition of 2.5  $\mu$ l of the above RNA

sample.

Reaction Solution Composition (each concentration represents a concentration in a final reaction solution volume of 30  $\mu$ l)

- 5        60 mM Tris-HCl buffer (pH 8.6)  
         17 mM magnesium chloride  
         90 mM potassium chloride  
         39 U RNase Inhibitor  
         1 mM DTT
- 10       0.25  $\mu$ l of each dATP, dCTP, dGTP, dTTP  
         3.6 mM ITP  
         3.0  $\mu$ l of each ATP, CTP, GTP, UTP  
         0.16  $\mu$ M first oligonucleotide  
         1.0  $\mu$ M second oligonucleotide
- 15       1.0  $\mu$ M third oligonucleotide  
         13% DMSO  
         Distilled water for adjusting volume

(3) RNA amplification reactions were carried out using the oligonucleotide sequences listed in Table 3, as the first, second and third oligonucleotides. Solutions were prepared so that the combinations of the first, second and third oligonucleotides would be those as listed in Table 3.

- (4) After incubating the above reaction solutions for 5 minutes at 41°C, 4.2  $\mu$ l of an enzyme solution having the following composition was added.

Composition of Enzyme Solution (each figure represents the amount in a final reaction solution volume of 30  $\mu$ l)

- 30       1.7% sorbitol  
         3  $\mu$ g bovine serum albumin  
         142 U T7 RNA polymerase (Gibco)  
         8 U AMV-Reverse Transcriptase (Takara Shuzo Co. Ltd.)

Distilled water for adjusting volume

(5) Subsequently, the PCR tubes were incubated at 41°C for 30 minutes. In order to identify the RNA amplified portion after the reaction, agarose gel (agarose concentration 4%) electrophoresis was performed. Dyeing following the electrophoresis was performed with SYBR Green II (Takara Shuzo Co. Ltd.). When an oligonucleotide probe binds to the specific portion of the target RNA, the RNA portion between the second and third oligonucleotide is amplified and, thereby, a characteristic band could be observed.

The results of the electrophoresis are shown in Figs. 3 and 4 (black and white inverted). The lengths of the specific bands amplified in this reaction are shown in Table 3. Since specific bands were confirmed in any of the combinations shown in Table 3, it was demonstrated that these oligonucleotides are effective in detecting VT1 RNA.

Table 3

Combination	1st Oligo-nucleotide Probe	2nd Oligo-nucleotide Probe	3rd Oligo-nucleotide Probe	Amplification Product Length (Base)
(a)	5S	5F	6R	141
(b)	6S	6F	7R	166
(c)	6S	6F	8R	346
(d)	7S	7F	8R	191
(e)	7S	7F	9R	281
(f)	8S	8F	9R	101

Table 3 shows the combinations of first, second and third oligonucleotides used in this example, as well as the chain lengths of the amplified specific bands resulted from the RNA amplification reaction using these combinations. The 3' end hydroxyl group of each first oligonucleotide base sequence was aminated. In each second oligonucleotide base sequence, the region of the 1st "A" to the 22nd "A" from the 5' end corresponds to the T7 promoter region, and the subsequent region from the 23rd "G" to the 28th "A" corresponds to the enhancer

sequence.

First oligonucleotide

- 5S (SEQ. ID. No. 27)
- 6S (SEQ. ID. No. 28)
- 5 7S (SEQ. ID. No. 29)
- 8S (SEQ. ID. No. 30)

Second oligonucleotide

- 5F (SEQ. ID. No. 36)
- 6F (SEQ. ID. No. 37)
- 10 7F (SEQ. ID. No. 38)
- 8F (SEQ. ID. No. 39)

Third oligonucleotide

- 6R (SEQ. ID. No. 2)
- 7R (SEQ. ID. No. 26)
- 15 8R (SEQ. ID. No. 3)
- 9R (SEQ. ID. No. 4)

Example 4

RNA amplification reactions were carried out using the oligonucleotides which specifically bind to VT2 RNA.

- 20 (1) As described in example 2, VT2 standard RNA was diluted to  $10^4$  copies/2.5  $\mu$ l and  $10^3$  copies/2.5  $\mu$ l with an RNA diluent (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 U/ $\mu$ l RNase Inhibitor (Takara Shuzo Co. Ltd.), 5 mM DTT). In the control test sections (negative), only the
- 25 diluent was used.

- (2) 23.3  $\mu$ l of a solution having the following composition was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tube™, Perkin-Elmer Co. Ltd.), followed by addition of 2.5  $\mu$ l of the above RNA
- 30 sample.

Reaction Solution Composition (each concentration represents a concentration in a final reaction solution volume of 30  $\mu$ l)

- 60 mM Tris-HCl buffer (pH 8.6)
- 35 17 mM magnesium chloride

90 mM potassium chloride  
39 U RNase Inhibitor  
1 mM DTT  
0.25  $\mu$ l of each dATP, dCTP, dGTP, dTTP  
5 3.6 mM ITP  
3.0  $\mu$ l of each ATP, CTP, GTP, UTP  
0.16  $\mu$ M first oligonucleotide  
1.0  $\mu$ M second oligonucleotide  
1.0  $\mu$ M third oligonucleotide

10 13% DMSO  
Distilled water for adjusting volume

(3) RNA amplification reactions were carried out using the oligonucleotide sequences listed in Table 4, as the first, second and third oligonucleotides. Solutions  
15 were prepared so that the combinations of the first, second and third oligonucleotides would be those as listed in Table 4.

(4) After incubating the above reaction solutions for 5 minutes at 41°C, 4.2  $\mu$ l of an enzyme solution  
20 having the following composition was added.

Composition of Enzyme Solution (each figure represents the amount in a final reaction solution volume of 30  $\mu$ l)

1.7% sorbitol  
25 3  $\mu$ g bovine serum albumin  
142 U T7 RNA polymerase (Gibco)  
8 U AMV-Reverse Transcriptase (Takara Shuzo Co. Ltd.)  
Distilled water for adjusting volume

30 (5) Subsequently, the PCR tubes were incubated at 41°C for 30 minutes. In order to identify the RNA amplified portion after the reaction, agarose gel (agarose concentration 4%) electrophoresis was performed. Dyeing following the electrophoresis was performed with  
35 SYBR Green II (Takara Shuzo Co. Ltd.). When an

oligonucleotide probe binds to the specific portion of the target RNA, the RNA portion between the second and third oligonucleotide is amplified, thereby a characteristic band could be observed.

The results of the electrophoresis are shown in Figs. 5 to 7 (black and white inverted). The lengths of the specific bands amplified in this reaction are shown in Table 4. Since specific bands were confirmed in any of the combinations shown in Table 4, it was demonstrated that these oligonucleotides are effective in detecting VT1 RNA.

Table 4

Combination	1st Oligo-nucleotide Probe	2nd Oligo-nucleotide Probe	3rd Oligo-nucleotide Probe	Amplification Product Length (Base)
(g)	B2S	B2F	B4R	274
(h)	B3S	B3F	B4R	116
(i)	B3S	B3F	B5R	187
(j)	B4S	B4F	B7R	321
(k)	B5S	B5F	B7R	250
(l)	B5S	B5F	B8R	298
(m)	B7S	B7F	B9R	123

Table 4 shows the combinations of first, second and third oligonucleotides used in this example, as well as the chain lengths of the amplified specific bands resulted from the RNA amplification reaction using these combinations. The 3' end hydroxyl group of each first oligonucleotide base sequence was aminated. In each second oligonucleotide base sequence, the region of the 1st "A" to the 22nd "A" from the 5' end corresponds to the T7 promoter region, and the subsequent region from the 23rd "G" to the 28th "A" corresponds to the enhancer sequence.

First oligonucleotide

B2S (SEQ. ID. No. 31)

B3S (SEQ. ID. No. 32)

B4S (SEQ. ID. No. 33)

B5S (SEQ. ID. No. 34)

B7S (SEQ. ID. No. 35)



Second oligonucleotide

- B2F (SEQ. ID. No. 40)
- B3F (SEQ. ID. No. 41)
- B4F (SEQ. ID. No. 42)
- 5 B5F (SEQ. ID. No. 43)
- B7F (SEQ. ID. No. 44)

Third oligonucleotide

- B4R (SEQ. ID. No. 8)
- B5R (SEQ. ID. No. 9)
- 10 B7R (SEQ. ID. No. 10)
- B8R (SEQ. ID. No. 11)
- B9R (SEQ. ID. No. 12)

Example 5

- Combinations of oligonucleotide primers according to the present invention were used for specific detection of different initial copy numbers of the target VT2 RNA.
- 15

- (1) As described in example 2, VT2 standard RNA was diluted with an RNA diluent (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 U/ $\mu$ l RNase Inhibitor (Takara Shuzo Co. Ltd.), 5 mM DTT) to concentrations ranging from  $10^5$  copies/2.5  $\mu$ l to  $10^1$  copies/2.5  $\mu$ l. In the control testing sections, only the diluent was used (Negative).
- 20

- (2) 23.3  $\mu$ l of a reaction solution having the composition shown below was dispensed into 0.5 ml volume PCR tubes (Gene Amp Thin-Walled Reaction Tube™, Perkin-Elmer) followed by addition of 2.5  $\mu$ l of the above RNA sample.
- 25

Reaction Solution Composition (each concentration represents that in a final reaction solution of 30  $\mu$ l)

- 30 60 mM Tris-HCl buffer (pH 8.6)
- 17 mM magnesium chloride
- 150 mM potassium chloride
- 39 U RNase Inhibitor
- 1 mM DTT
- 35 0.25 mM each of dATP, dCTP, dGTP and dTTP

3.6 mM ITP

3.0 mM each of ATP, CTP, GTP and UTP

0.16  $\mu$ M first oligonucleotide (5S shown in Table 4,  
wherein its 3' end is aminated)

5 1.0  $\mu$ M second oligonucleotide (5F shown in Table 4)

1.0  $\mu$ M third oligonucleotide (7R shown in Table 4)

25 nM intercalator fluorescent pigment-labeled  
oligonucleotide (SEQ. ID. No. 25, labeled with an  
intercalator fluorescent pigment at the phosphorous atom  
10 between the 12th "T" and the 13th "A" from the 5' end,  
and modified with a glycol group at its 3' end hydroxyl)

13% DMSO

Distilled water for adjusting volume

(3) After incubating the above reaction solution  
15 for 5 minutes at 41°C, 4.2  $\mu$ l of an enzyme solution  
having the following composition and pre-incubated for  
2 minutes at 41°C was added.

Enzyme Solution Composition (each concentration  
represents that in a final reaction solution of 30  $\mu$ l)

20 1.7% sorbitol

3  $\mu$ g bovine serum albumin

142 U T7 RNA polymerase (Gibco)

8 U AMV-Reverse Transcriptase (Takara Shuzo Co.  
Ltd.)

25 Distilled water for adjusting volume

(4) The PCR tube was then incubated at 41°C using a  
direct-measuring fluorescence spectrophotometer equipped  
with a temperature-controller, and the reaction solution  
was periodically measured at an excitation wavelength of  
30 470 nm and a fluorescent wavelength of 510 nm.

Fig. 8 (A) shows the time-course changes in the  
fluorescence increase ratio (fluorescence intensity at  
predetermined time/background fluorescence intensity) of  
the sample, where enzyme was added at 0 minutes. Fig. 8  
35 (B) shows the relationship between the logarithm of the

initial RNA amount and the rise time (time at which the relative fluorescence reaches the negative sample's average value plus 3 standard deviations; i.e., the time to reach a ratio of 1.2). The initial RNA amount was  
5 between  $10^1$  copies/test and  $10^5$  copies/test.

Fig. 8 shows that  $10^1$  copies were detected at approximately 20 minutes. A fluorescent profile and calibration curve depending on the initial concentration of the labeled RNA were obtained, indicating that it is  
10 possible to quantify the VT2 RNA present in unknown samples. This demonstrated that speedy, highly sensitive detection of VT2 RNA is possible using this method.

As explained above, the oligonucleotide provided by the present invention complementarily binds to the  
15 intramolecular structure-free region of VT1 RNA or VT2 RNA. By using this oligonucleotide, it is possible to detect an RNA by a process carried out under a relatively low and constant temperature, without the need of an operation which destroys the intramolecular structure of  
20 an RNA by heat-degradation so as to improve the primer binding efficiency. As a result, by use of the oligonucleotide according to the present invention, it would be possible to provide an RNA detection method which is speedy, simple, and even suitable for  
25 automation.

It will be appreciated by those skilled in the art that while the invention has been described above in connection with particular embodiments and examples, the invention is not necessarily so limited and that numerous  
30 other embodiments, examples, uses, modifications and departures from the embodiments, examples and use may be made without departing from the inventive scope of this application.